# Effect of Candida antarctica Lipase B Immobilization on the Porous Structure of the Carrier

Miletic, Nemanja; Vukovic, Zorica; Nastasovic, Aleksandra; Loos, Katja; Miletić, Nemanja; Vuković, Zorica; Nastasović, Aleksandra

*Published in:*
Macromolecular Bioscience

*DOI:*
10.1002/mabi.201100127

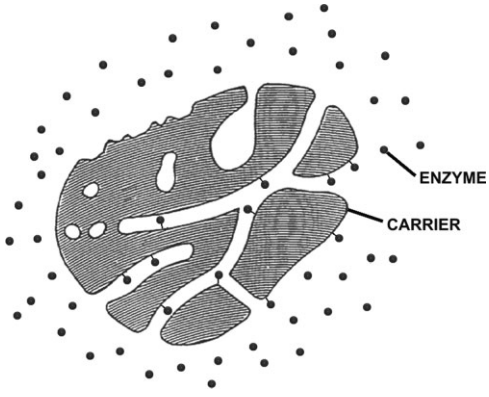


*Publication date:*
2011

# Effect of *Candida antarctica* Lipase B Immobilization on the Porous Structure of the Carrier

Nemanja Miletić, Zorica Vuković, Aleksandra Nastasović, Katja Loos*

A series of poly(GMA-*co*-EGDMA) resins with identical composition but varying particle sizes, pore radii, specific surface areas and specific volumes are studied to assess how *Candida antarctica* lipase B immobilization affects the porosity of the copolymer particles. Mercury porosimetry reveals a significant change in the average pore size (up to 6.1-fold), the specific surface area (up to 3.2-fold) and the specific volume (up to 2.1-fold) of the epoxy resin. A similar behaviour is observed for glutaraldehyde-modified epoxy resins. The influences of the resin porosity properties on the loading of *Candida antarctica* lipase B during immobilization and on the hydrolytic activity (hydrolysis of *p*-nitrophenyl acetate) of the immobilized lipase are studied.




## Introduction

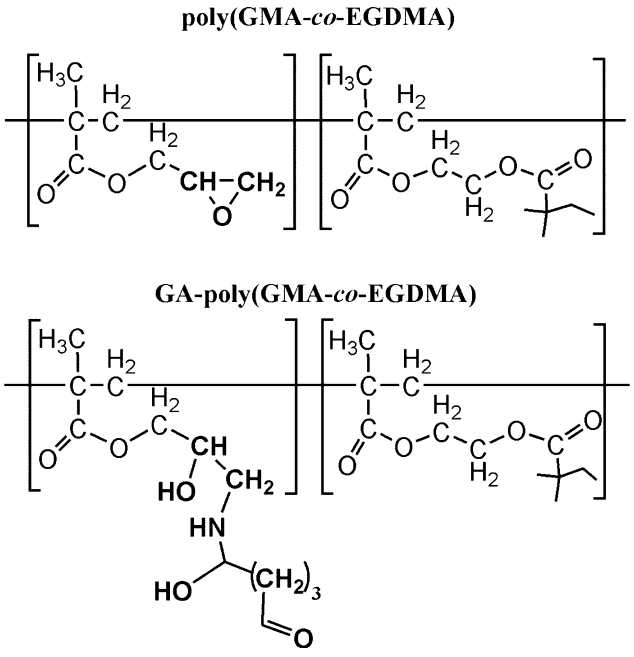
Macroporous copolymers are frequently used for the preparation of various types of ion-exchange resins, as carriers for some types of chromatography, as adsorbents, as supports for classical catalysts or enzymes in biosynthesis and as membranes for different purposes.[1–4] It has been shown that the macroporous copolymer poly[(glycidyl methacrylate)-*co*-(ethylene glycol dimethacrylate)] [poly(GMA-*co*-EGDMA)] can be synthesized by suspension polymerization when an inert component [mixture of cyclohexanol as solvent and aliphatic alcohol as non-solvent (porogen) for copolymer] is present in the monomer mixture.[5–7] The composition and amount of inert component have the largest influence on the porous structure of poly(GMA-*co*-EGDMA).

Natural enzymes seldom have the features adequate to be used as industrial catalysts in organic synthesis. An important route to improving enzyme performance in non-natural environments is to immobilize them.[8,9] In this study, *Candida antarctica* lipase B (Cal-B) is chosen as a versatile enzyme for enantio- and regioselective transformations.[10–14] Poly(GMA-*co*-EGDMA) (an eupergit-like copolymer) has been previously used for enzyme immobilization.[15–17] These copolymers offer a large surface area, a hydrophilic microenvironment, active epoxide groups and the possibility of obtaining targeted porous structure.[7] These varieties of the porous structures that can be obtained offer the possibility of finding copolymer with suitable

N. Miletić, K. Loos
Polymer Chemistry Department, Zernike Institute for Advanced Materials, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands
E-mail: K.U.Loos@rug.nl
N. Miletić
Fruit Research Institute, Kralja Petra I no 9, 32000 Čačak, Serbia
Z. Vuković
ICTM – Center for Catalysis and Chemical Engineering, University of Belgrade, Njegoševa 12, 11000 Belgrade, Serbia
A. Nastasović
ICTM – Center for Chemistry, University of Belgrade, Studentski trg 12-16, 11001 Belgrade, Serbia




DOI: 10.1002/mabi.201100127




*Scheme 1.* Chemical structures of poly(GMA-*co*-EGDMA) and GA-poly(GMA-*co*-EGDMA).

surface characteristics for the stabilization of an investigated enzyme.[18]

The influence of enzyme immobilization on the properties of macroporous beads (such as specific volume, pore radius and specific surface area) has not been studied so far. However, it is important to correlate these properties to the success of enzyme immobilization not only before but also after the immobilization process, as there might be important changes in these structural features invoked by process. To elucidate this we investigated the effect of Cal-B immobilization on the porous structure of poly(GMA-*co*-EGDMA) and glutaraldehyde (GA)-modified poly(GMA-*co*-EGDMA) [GA-poly(GMA-*co*-EGDMA)] (Scheme 1). It is shown that the porous properties of the carrier have the strongest influence on the enzyme loading and the enzyme activity.

## Experimental Section

### Materials

Cal-B in the form of a dried powder was purchased from Codexis (Jülich, Germany). Poly(*N*-vinylpyrrolidone) was purchased from Fluka and used as supplied. All other chemicals were used as supplied by Sigma-Aldrich.

### Preparation of Carrier

The samples of poly(GMA-*co*-EGDMA) with different porosity parameters were prepared by a radical suspension polymerization as described in refs.[5–7,19–21] In all experiments, 80.5 g of monomer phase, which contained monomer mixture (20.7 g of GMA and 13.8 g of EGDMA), azoisobutyronitrile (AIBN) as an initiator (0.8 g) and 45.2 g of inert component (40.7 g of cyclohexanol and 4.5 g of tetradecanol or hexadecanol for samples SGE-10/14 and SGE-10/16, respectively, and 36.2 g of cyclohexanol and 9.0 g of tetradecanol for sample SGE-20/14) was suspended in 240.0 g of a 1 wt% aqueous solution of poly(*N*-vinyl pyrrolidone), PVP. In the labels of copolymer samples, letter S designates suspension copolymerization, G and E denote the monomers (GMA and EGDMA, respectively). The first number in a sample labels gives the fraction of aliphatic alcohol in the inert component (w/w) and the second one the number of C atoms in the aliphatic alcohol. d1, d2, d3, d4 indicate the particle diameter sizes 630–300, 300–150, 150–100 and $<100\ \mu m$, respectively.

The copolymerization was carried out at 70 °C for 2 h and then at 80 °C for 6 h with a stirring rate of 200 rpm. After completion of the reaction, the copolymer particles were washed with water and ethanol, kept in ethanol for 12 h and then dried in a vacuum oven at 45 °C for 24 h.

### Glutaraldehyde Modification of the Epoxy Carrier

The modification of the epoxy carrier, consisting first of the modification of poly(GMA-*co*-EGDMA) with ammonia (AMM, amination), and second the GA activation of the aminated particles, was performed based on the method used by Bilici et al.[22]

### Porosity Measurements

The pore size distributions were determined by mercury porosimetry (Carlo Erba 2000, software Milestone 200). The samples were dried at 50 °C for 8 h and degassed at room temperature and a pressure of 0.5 Pa for 2 h. The values of specific pore volume, $V_S$, and pore radius that corresponds to half of the pore volume, $r_{V/2}$, were read from cumulative pore distribution curves. The values of specific surface area, $S_{Hg}$, were calculated on the basis of cylindrical pore model as described in literature.[23]

### Enzyme Immobilization

The copolymer beads were added to a Cal-B solution ($6.67\ mg \cdot mL^{-1}$) in phosphate-buffered saline (PBS) pH = 6.8. The ratio of copolymer to Cal-B was 4:1 in all experiments. The samples were incubated in a rotary shaker at 200 rpm at 30 °C. After 24 h the solution was removed by filtering and the resulting immobilized Cal-B was washed with PBS buffer and distilled water, until no protein was detectable any more in the washing solution. Supernatant and washing solutions were collected and using the bicinchoninic acid (BCA) protein assay, the amount of enzyme that is immobilized could be estimated. The resulting resins with immobilized Cal-B were freeze-dried for 48 h, prior to use. Enzyme loading is defined herein as the weight of Cal-B that is immobilized per total weight of carrier.

### Enzyme Hydrolytic Activity

A 1,4-dioxane solution (5.0 mL) containing *p*-nitrophenyl acetate (*p*NPA) ($40.0 \times 10^{-3}$ M) and methanol ($80.0 \times 10^{-3}$ M) was added to

20 mL vials containing 0.772 mg of enzyme. The assay reactions were carried out for 50 min at 35 °C (300 rpm) and were terminated by removal of the enzyme by filtration. The concentration of the reaction product *p*-nitrophenol (*p*NP) was determined by UV/VIS spectroscopy (PYE UNICAM SP8-200 UV/VIS spectrophotometer) at the $\lambda_{max}$ of *p*NP (304 nm). Enzyme hydrolytic activities for Cal-B immobilized on poly(GMA-*co*-EGDMA) and on GA-poly(GMA-*co*-EGDMA) are defined herein as the amount of *p*NPA hydrolyzed in 1,4-dioxane per unit of weight of enzyme per time (nmol $p\text{NP} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$).

## Results and Discussions

### Porous Structure of Epoxy Carrier

A series of poly(GMA-*co*-EGDMA) resins with identical compositions (60% of GMA) were synthesized, and porosity parameters were determined by mercury intrusion porosimetry, invoking cylindrical pore model. From the pore size distribution curves presented in Figure 1, it can be concluded that composition and amount of inert component cause considerable alteration of porosity parameters of poly(GMA-*co*-EGDMA).

According to previous studies,[5,7] there is a slight increase in specific volume and pore radius and a decrease in specific surface area when the number of carbon atoms in the aliphatic alcohol is increased while keeping the amount of alcohol in the inert component constant. The same trend can be observed when a higher amount of aliphatic alcohol in the inert component is used. In our case (Figure 1), the same trend is noticed, except for the resin SGE-20/16-d1, where unexpectedly a pore radius of 30 nm is obtained. Indeed, the sample synthesized with hexadecanol (SGE-20/16) manifests different porous features. For example, it was observed the fraction with diameter in the range of 300–150 μm do not have spherical shape (sample SGE-20/16-d2).[7]

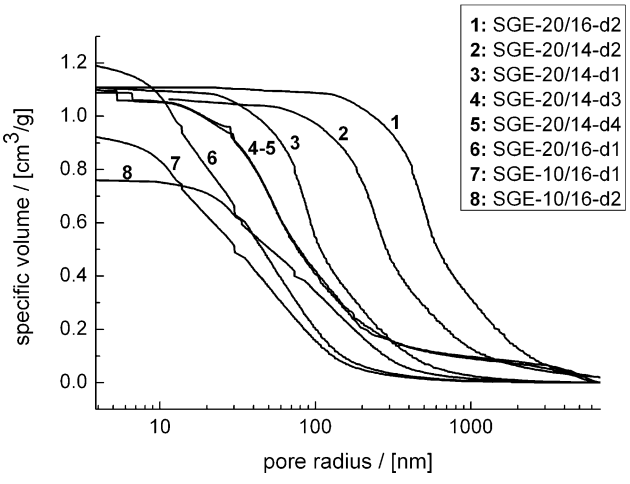



*Figure 1.* Cumulative pore size distribution curves for series of poly(GMA-*co*-EGDMA).

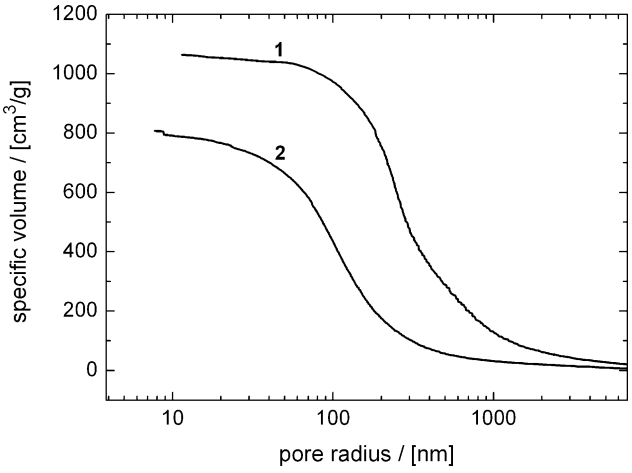



*Figure 2.* Cumulative pore size distribution curves for resin SGE-20/14-d1 and resin SGE- before (1) and after lipase immobilization (2).

### Enzyme Immobilization on Poly(GMA-*co*-EGDMA)

Porosity measurements of epoxy resins were performed before and after enzyme immobilization. Figure 2 represents the cumulative pore size distribution curves for resin SGE-20/14-d2 before and after lipase immobilization as an example. All other results are summarized in Table 1. It becomes obvious that enzyme immobilization changes the porosity properties of the carrier. Therefore, it can be concluded that the enzymes adhere firmly to the resin. For resins SGE-10/16-d1, SGE-10/16-d2, SGE-20/14-d1, SGE-20/14-d3, SGE-20/14-d4, SGE-20/16-d1, enzyme immobilization is followed by a corresponding significant increase in the average pore radius, and decrease in the specific surface area and specific volume (Table 1). A different situation is observed for the resins SGE-20/14-d2 and SGE-20/16-d2: enzyme immobilization causes a decrease in the average pore radius and specific volume, and a significant increase in the specific surface area (Table 1). These results are caused by the huge differences in average pores size radius in the various resins. Namely, the resins SGE-20/14-d2 and SGE-20/16-d2 have a quite high value of the average pore radius, 270 and 560 nm, respectively, while other resins have a much smaller average pore size radius (from 30 to 92 nm). Since Cal-B is a globular type protein with the approximate dimensions of $3.0 \times 4.0 \times 5.0$ nm,[24] it can be expected for the carrier with low average pore size radius that enzyme molecules are being immobilized primarily into the smallest possible pores, resulting in a shift of the average pore radius to a higher value and a dramatic decrease of the specific surface area. On the other hand, for

*Table 1.* Porosity parameters of poly(GMA-*co*-EGMDA) copolymer particles before and after Cal-B immobilization, enzyme loading and enzyme activity.

| Sample | Treatment | Average pore radius[a)] [nm] | Specific surface area[a)] [$m^2 \cdot g^{-1}$] | Specific volume[a)] [$cm^3 \cdot g^{-1}$] | Enzyme loading[a)] [$\mu g \cdot mg^{-1}$] | Hydrolytic activity[a)] [nmol *p*NP $\cdot$ $min^{-1}$ $\cdot$ (mg Cal-B)$^{-1}$] |
|---|---|---|---|---|---|---|
| SGE-10/16-d1 | BI[b)] | 30 ± 1.2 | 82.0 ± 4.1 | 0.923 ± 0.05 | | |
| | AI[c)] | 44 ± 1.4 | 48.3 ± 3.3 | 0.448 ± 0.05 | 222.7 ± 3.2 | 1065.4 ± 22.2 |
| SGE-10/16-d2 | BI | 87 ± 3.5 | 36.0 ± 1.8 | 0.755 ± 0.04 | | |
| | AI | 95 ± 2.3 | 30.1 ± 1.7 | 0.434 ± 0.04 | 220.8 ± 2.2 | 2125.0 ± 19.0 |
| SGE-20/14-d1 | BI | 92 ± 4.6 | 36.0 ± 1.8 | 1.111 ± 0.06 | | |
| | AI | 115 ± 3.3 | 30.6 ± 1.9 | 1.045 ± 0.06 | 178.2 ± 3.6 | 1928.4 ± 31.2 |
| SGE-20/14-d2 | BI | 270 ± 13.5 | 27.6 ± 1.4 | 1.040 ± 0.05 | | |
| | AI | 48 ± 6.3 | 48.8 ± 1.9 | 0.807 ± 0.04 | 207.8 ± 4.7 | 2775.0 ± 69.8 |
| SGE-20/14-d3 | BI | 59 ± 3.0 | 46.7 ± 2.3 | 1.088 ± 0.05 | | |
| | AI | 74 ± 2.6 | 31.2 ± 2.9 | 0.913 ± 0.05 | 166.9 ± 2.7 | 4534.9 ± 88.9 |
| SGE-20/14-d4 | BI | 48 ± 2.4 | 55.2 ± 2.8 | 1.100 ± 0.06 | | |
| | AI | 74 ± 2.2 | 35.5 ± 2.2 | 0.967 ± 0.07 | 157.1 ± 2.8 | 5027.2 ± 33.6 |
| SGE-20/16-d1 | BI | 30 ± 1.2 | 106.0 ± 5.3 | 1.191 ± 0.06 | | |
| | AI | 110 ± 2.3 | 32.7 ± 6.8 | 0.952 ± 0.05 | 184.8 ± 1.2 | 1801.8 ± 19.0 |
| SGE-20/16-d2 | BI | 560 ± 28.0 | 13.2 ± 0.7 | 1.125 ± 0.06 | | |
| | AI | 92 ± 2.6 | 37.8 ± 2.5 | 0.926 ± 0.05 | 172.1 ± 1.2 | 2875.6 ± 29.3 |

[a)]Standard deviations were calculated from three replicate experiments; [b)]Before immobilization; [c)]After immobilization.

the carriers with high values of the average pore radius (SGE-20/14-d2 and SGE-20/16-d2), it becomes obvious that enzyme molecules are being immobilized uniformly throughout the carrier pores, resulting in a large decrease in the average pore radius and increase in the specific surface area. The specific volume, i.e. the volume of the mercury penetrated in the sample, is decreased in all resins after enzyme immobilization due to the firmly attached enzymes within the pores that reduce the pore volume.

It was observed that loading and activity of Cal-B immobilized on poly(GMA-*co*-EGDMA) copolymer particles strongly depends on the porosity characteristics of the carrier.

Cal-B loading depends particularly on pore size and specific surface area (Table 1). Increasing the pore size of the 300–150 μm beads from 87 (SGE-10/16-d2) to 270 (SGE-20/14-d2) and 560 nm (SGE-20/16-d2) resulted in a decrease in enzyme loading (220.8, 207.8, and 172.1 $\mu g \cdot mg^{-1}$). The same tendency was observed for increasing the pore size of the 630–300 μm beads from 30 nm (SGE-10/16-d1 and SGE-20/16-d1) to 92 nm (SGE-20/14-d1). On the other hand, increasing the specific surface area of the 630–300 μm beads, with identical pore size of 30 nm, from 82.0 (SGE-10/16-d1) to 106.0 $m^2 \cdot g^{-1}$ (SGE-20/16-d1) resulted in a decrease in enzyme loading from 222.7 to 184.8 $\mu g \cdot mg^{-1}$. These results are attributed to the fact that the greater amount of enzyme is bound onto polymers with broad pore size distribution and that the pore size distribution can be very different for the copolymers with identical average pore size or specific surface area.[25,26]

Table 1 illustrates that *p*NPA hydrolysis is also strongly dependent on the porosity parameters of the resin. Increasing the pore size of the 300–150 μm beads from 87 (SGE-10/16-d2) to 270 (SGE-20/14-d2) and 560 nm (SGE-20/16-d2) resulted in a large increase in the hydrolytic activity (from 2125.0 to 2775.0 and 2875.6). The same trend is noticed when increasing the pore size of 630–300 μm beads from 30 to 92 nm (SGE-10/16-d1 and SGE-20/14-d1, respectively). Resins with the pore radius of 87, 270 and 560 nm are big enough to overcome diffusion limitation. On the other hand, increase in the pore size of the resin corresponds to an increase in the percent area of beads at which Cal-B is found, so substrate and product diffusion to enzyme regions are facilitated.[27] Furthermore, increasing the specific surface area of the 630–300 μm beads, with identical pore size of 30 nm, from 82.0 (SGE-10/16-d1) to 106.0 $m^2 \cdot g^{-1}$ (SGE-20/16-d1) resulted in a large increase in the hydrolytic activity (from 1065.4 to 1801.8). Increase in

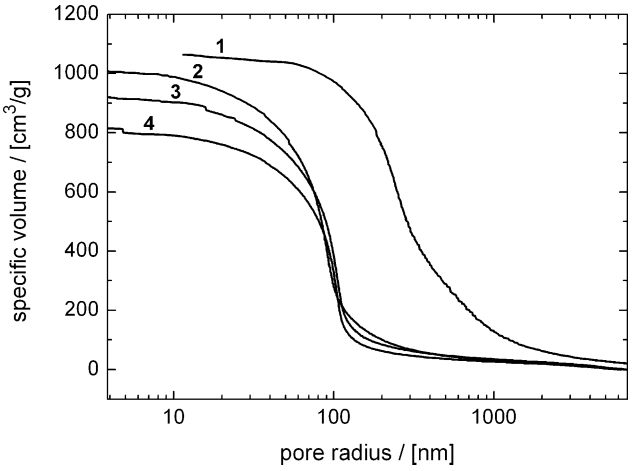



*Figure 3.* Cumulative pore size distribution curves for resin SGE-20/14-d1 (1), after modification with AMM (2), adequate activation with GA (3) and after enzyme immobilization (4).

the specific surface area alleviates substrate approach to the immobilized enzyme zones.

### Enzyme Immobilization on GA-poly(GMA-*co*-EGDMA)

Porosity measurements were performed after every modification step in the GA modification procedure, as well as after lipase immobilization on modified carrier. Figure 3 represents the cumulative pore size distribution curves for resin SGE-20/14-d2 as an example: (i) before modification, (ii) after modification with AMM, (iii) after activation of aminated particles with GA and (iv) after lipase immobilization on activated carrier. Every step is followed by a corresponding significant change in porosity parameters, which is a strong evidence for a successful modification process. For the carriers with high values of the average pore radius (SGE-20/14-d2 and SGE-20/16-d2), modification with AMM resulted in a significant decrease in the average pore size radius and an increase in the specific surface area (results for SGE-20/14-d2 are given in Table 2 as an example). On the other hand, for the resins with a low value of the average pores radius the same modification step caused a completely opposite effect (results for SGE-20/14-d1 are given in Table 2 as an example). Most likely these behaviours follow the same principles as already above observed during enzyme immobilization. For all resins, activation with GA was followed by a corresponding moderate increase in average pore size radius and decrease in specific surface area (Table 2). In all samples, the specific volume is decreased after every modification step as well as after lipase immobilization which can be expected since novel molecules are being incorporated in the pores and therefore reduce the pore volume.

Altering the porosity structure is also noticed after enzyme immobilization, following the above-mentioned regularities.

Determination of enzyme loading and activity of Cal-B immobilized on GA-poly(GMA-*co*-EGDMA) (Table 3) clearly revealed that Cal-B activity is significantly improved by activation of poly(GMA-*co*-EGDMA) with GA. On the other hand, all modified samples have lower enzyme loadings compared to the initial ones.

It was already proved that the amount of GA used for modification plays an important role for the enzyme loading and activity.[28] Increasing the amount of GA used for modification resulted in an increase of the enzyme

*Table 2.* Porosity parameters of poly(GMA-*co*-EGDMA) copolymer particles before modification, after activation with AMM, after modification with GA and after Cal-B immobilization.

| Sample | Treatment | Average pore radius[a)] [nm] | Specific surface area[a)] [$m^2 \cdot g^{-1}$] | Specific volume[a)] [$cm^3 \cdot g^{-1}$] |
|---|---|---|---|---|
| SGE-20/14-d1 | BM[b)] | 92 ± 4.6 | 36.0 ± 1.8 | 1.111 ± 0.06 |
| | M-AMM[c)] | 143 ± 6.1 | 33.6 ± 1.7 | 1.110 ± 0.06 |
| | AC-GA[d)] | 145 ± 4.2 | 26.2 ± 1.2 | 0.934 ± 0.06 |
| | AI[e)] | 151 ± 4.0 | 21.8 ± 1.1 | 0.907 ± 0.06 |
| SGE-20/14-d2 | BM | 270 ± 13.5 | 27.6 ± 1.4 | 1.040 ± 0.05 |
| | M-AMM | 88 ± 4.4 | 38.6 ± 1.8 | 1.007 ± 0.06 |
| | AC-GA | 92 ± 5.0 | 27.6 ± 1.7 | 0.921 ± 0.05 |
| | AI | 107 ± 3.0 | 21.3 ± 1.3 | 0.815 ± 0.05 |

[a)]Standard deviations were calculated from three replicate experiments; [b)]Before modification; [c)]Modification with AMM; [d)]Activation with GA; [e)]After immobilization.

*Table 3.* Enzyme loading and activity of Cal-B immobilized on GA-poly(GMA-*co*-EGDMA).

| Sample | Modification | Activation | Enzyme loading[a)] [$\mu g \cdot mg^{-1}$] | Hydrolytic activity [$nmol\ pNP \cdot min^{-1} \cdot (mg\ Cal\text{-}B)^{-1}$][a)] |
|---|---|---|---|---|
| SGE-10/16-d1 | AMM | GA | $192.31 \pm 2.8$ | $1\,311.4 \pm 39.0$ |
| SGE-10/16-d2 | AMM | GA | $185.41 \pm 2.2$ | $2\,883.9 \pm 32.0$ |
| SGE-20/14-d1 | AMM | GA | $151.53 \pm 4.4$ | $2\,762.8 \pm 40.6$ |
| SGE-20/14-d2 | AMM | GA | $161.24 \pm 2.9$ | $3\,121.6 \pm 32.1$ |
| SGE-20/14-d3 | AMM | GA | $155.20 \pm 3.5$ | $5\,716.1 \pm 21.6$ |
| SGE-20/14-d4 | AMM | GA | $135.24 \pm 3.3$ | $7\,810.1 \pm 28.1$ |
| SGE-20/16-d1 | AMM | GA | $155.81 \pm 8.1$ | $2\,290.0 \pm 62.2$ |
| SGE-20/16-d2 | AMM | GA | $148.52 \pm 5.2$ | $3\,409.0 \pm 55.0$ |

[a)]Standard deviation values were calculated from three replicate experiments.

loading, while Cal-B activity primary increases showing the maximum, and subsequently decreases.[28]

## Conclusion

The results obtained show that it is possible to influence the parameters of pore structure (average pore radius, specific surface area and specific volume) of poly(GMA-*co*-EGDMA), obtained by suspension polymerization by changing type and ratio of aliphatic alcohol in inert component. Increasing the number of carbon atoms in the aliphatic alcohol resulted in a slight increase in specific volume and pore radius, and a decrease in specific surface area.

It was clearly shown that immobilization of Cal-B on poly(GMA-*co*-EGDMA) significantly changed the porous structure of the carrier. Depending on the average pore size radius of poly(GMA-*co*-EGDMA), different behaviour during immobilization is observed. Namely, enzyme immobilization on resins with a high value for the average pore radius (from 270 to 560 nm) resulted in a large decrease in the average pore radius, and an increase in the specific surface area, most likely due to the uniform enzyme immobilization throughout the carrier bead. A completely opposite effect is observed when performing enzyme immobilization on resins with low value for average pore radius (from 30 to 92 nm), due to the occupation of the smallest pores by immobilized enzymes.

It was clearly shown that the immobilized Cal-B activity strongly depends on the porosity characteristics of the carrier. Increasing the pore size of the 300–150 $\mu$m beads from 87 to 270 and 560 nm resulted in a large increase in the hydrolytic activity. Also, increasing the specific surface area of the 630–300 $\mu$m beads from 82.0 to 106.0 $m^2 \cdot g^{-1}$ added up in a large increase of hydrolytic enzyme activity. These behaviours were attributed to an increase in the regions where enzyme is immobilized and facilitating substrate diffusion to enzyme in resin with bigger pore size.

Modification of poly(GMA-*co*-EGDMA) with GA was also followed by a corresponding porosity alteration, as the evidence for successful modification procedure. We were able to prove that Cal-B activity is significantly improved by activation of poly(GMA-*co*-EGDMA) with GA.

Here we have shown for the first time that the process of immobilization of enzymes changes the properties of macroporous beads (such as specific volume, pore radius and specific surface area) tremendously. Therefore it is advisable to determine these important parameters after the immobilization process in further studies to avoid wrongful interpretation of the obtained data.

Acknowledgements: This work was partially supported by the Ministry of Science and Technological Development of the Republic of Serbia (Projects III 43009, and III 45001).

Received: April 2, 2011; Revised: June 6, 2011; Published online: August 12, 2011; DOI: 10.1002/mabi.201100127

Keywords: diffusion; enzymes; immobilization; lipase; macroporous copolymers

[1] A. Onjia, S. K. Milonjić, N. N. Jovanović, S. M. Jovanović, *React. Funct. Polym.* **2000**, *43*, 269.
[2] A. Hamerska-Dudra, J. Bryjak, A. W. Trochimczuk, *Enzyme Microb. Technol.* **2006**, *38*, 921.
[3] M. L. Zhang, Y. Sun, *J. Chromatogr., A* **2001**, *912*, 31.
[4] M. Hruby, J. Hradil, M. Benes, *J. React. Funct. Polym.* **2004**, *59*, 105.
[5] F. Švec, *Angew. Makromol. Chem.* **1986**, *114*, 39.

[6] D. Horak, F. Švec, M. Bleha, J. Kalal, *Angew. Makromol. Chem.* **1981**, *95*, 109.
[7] S. Jovanović, A. Nastasović, N. Jovanović, K. Jeremić, *Mater. Sci. Forum* **1996**, *214*, 155.
[8] W. Tischer, F. Wedekind, in: *Biocatalysis - from Discovery to Application* (Ed., W. D. Fressner), Springer, Berlin, Germany **1999**.
[9] G. R. Castro, T. Knubovets, *Crit. Rev. Biotechnol.* **2003**, *23*, 195.
[10] B. L. Zamost, H. K. Nielsen, R. L. Starnes, *J. Ind. Microbiol.* **1991**, *8*, 71.
[11] E. M. Anderson, M. Karin, O. Kirk, *Biocatal. Biotransform.* **1998**, *16*, 181.
[12] T. B. Nielsen, M. Ishii, O. Kirk, in: *Biotechnological Applications of Cold-adapted Organisms*, (Eds., R. Margesin, S. Schinner), Landes Bioscience, Austin, USA **1999**, pp. 49–61.
[13] Y. Naoshima, M. Kamezawa, T. Kimura, F. Okimoto, M. Watanabe, H. Tachibana, T. Ohtani, in: *Recent Research Developments in Organic & Bioorganic Chemistry* (Ed., S. G. Pandalai), Research Signpost, Trivandrum, India **2001**, pp. 1–16.
[14] F. Secundo, G. Carrea, *J. Mol. Catal. B: Enzym.* **2002**, *19*, 93.
[15] M. Marek, O. Valentova, J. Kas, *Biotechnol. Bioeng.* **1984**, *26*, 1223.
[16] R. Prodanović, S. Jovanović, Z. Vujičić, *Biotechnol. Lett.* **2001**, *23*, 1171.
[17] E. Katchalski-Katzir, D. M. Kraemer, *J. Mol. Catal. B: Enzym.* **2000**, *10*, 157.
[18] H. Takahashi, B. Li, T. Sasaki, C. Miyazaki, T. Kajino, S. Inagaki, *Chem. Mater.* **2000**, *12*, 3301.
[19] F. Švec, J. Hradil, J. Coupek, J. Kalal, *Angew. Makromol. Chem.* **1975**, *48*, 135.
[20] F. Švec, D. Horak, J. Kalal, *Angew. Makromol. Chem.* **1977**, *63*, 37.
[21] S. M. Jovanović, A. Nastasović, N. N. Jovanović, K. Jeremić, Z. Savić, *Angew. Makromol. Chem.* **1994**, *219*, 161.
[22] Z. Bilici, S. T. Camli, E. Unsal, A. Tuncel, *Anal. Chim. Acta* **2004**, *516*, 125.
[23] P. A. Webb, C. Orr, *Analytical Methods in Fine Particle Technology*, Micromeritics Instrument Corporation, Norcross, USA **1997**.
[24] J. Uppenberg, M. T. Hansen, S. Patkar, T. A. Jones, *Structure* **1994**, *2*, 293.
[25] N. Miletić, Z. Vuković, A. Nastasović, K. Loos, *J. Mol. Catal. B: Enzym.* **2009**, *56*, 196.
[26] A. Kotha, R. C. Raman, S. Ponrathnam, K. K. Kumar, J. G. Shewale, *React. Funct. Polym.* **1996**, *28*, 235.
[27] B. Chen, M. E. Miller, R. A. Gross, *Langmuir* **2007**, *23*, 6467.
[28] N. Miletić, R. Rohandi, Z. Vuković, A. Nastasović, K. Loos, *React. Funct. Polym.* **2009**, *69*, 68.